

## RESEARCH ARTICLE

# Phytochemical Screening by GC/MS with Isolation and Characterization of $\beta$ -sitosterol and Stigmasterol from Iraqi *Euonymus japonicus* L. Leaves Parts by Different Techniques

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## ABSTRACT

*Euonymus japonicus* L., often known as the spindle tree, is a Celastraceae family plant utilized in traditional medicine and as an attractive garden plant. The goal of this study was to identify and isolate the active compounds  $\beta$ -sitosterol and stigmasterol from the roots and leaves of *E. japonicus* by using gas chromatography/mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), and reverse phase-high performance liquid chromatography (RP-HPLC) were used to investigate the chemical composition of the leaves and root of *E. japonicus*. This confirms the presence of  $\beta$ -sitosterol and stigmasterol in the leaves and roots of *E. japonicus* L. plant. The structure of these chemicals was clearly defined by UV spectroscopic, FTIR spectroscopic, and TLC techniques, revealing similarity to  $\beta$ -sitosterol and stigmasterol.

**Keywords:** *Euonymus japonicus* L,  $\beta$ -sitosterol, Gas chromatography/Mass spectrometry (GC/MS), High-performance liquid chromatography (HPLC) and Preparative high-performance liquid chromatography (RP-HPLC).

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**Conflict of interest:** None

## INTRODUCTION

The Celastraceae family is native to tropical and subtropical regions of the world, including North Africa, South America, and much of East Asia, especially China.<sup>1,2</sup> The family has around 88 genera and 1300 plant species.<sup>3</sup> Resinous stems and leaves characterize these plants, which grow as small trees, shrubs, or lianas. They've been prized since antiquity for the therapeutic virtues of their extracts.<sup>4</sup>

*Euonymus*, often known as spindle or spindle tree, is a flowering plant genus in the Celastraceae family of staff vines. It contains approximately 170–180 deciduous and evergreen plants and small trees. They are usually found in East Asia. However, they can also be found in the Himalayas. We were compelled to explore several species of the genus *Euonymus* as part of our effort on bioactive secondary metabolite hunt and quality by design documentation of medicinal plants.<sup>5</sup>

*Euonymus japonicus* (Japanese *Euonymus*) is a Celastraceae family plant that is utilized as an ornamental garden plant. It was used to treat a variety of ailments as a form of traditional Chinese medicine.<sup>6</sup> Thus, utilizing sensitive instruments, a full profile of the phytochemicals in *E. japonicus* leaves and

root is required. With the goal of extracting  $\beta$ -sitosterol and stigmasterol from the n-hexane fraction of the leaves part for identification and quantification using HPLC, GC-MS, and thin layer chromatography (TLC) (Figure 1).<sup>7</sup>

This study aims to analyze  $\beta$ -sitosterol and stigmasterol from n-hexane fraction by using HPLC Further isolation of  $\beta$ -sitosterol and stigmasterol was accomplished using RPHPLC (Figure 2).

## MATERIALS AND METHODS

### Reagents and Materials

#### Plant Material Collection and Identification

The plant material of *E. japonicus* L. comprises of new leaves (aerial part) and roots. Cultivated in Iraq was manually gathered from zayona plant nurseries in Baghdad, Iraq, between October and November 2021. This plant was identified and confirmed by Dr. Israa Abdulrazaq from the College of Biology at the University of Baghdad. Before being extracted, the plants were dried at room temperature in the shade, crushed with an electronic mill, and weighed (Table 1 and Figure 3).

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## Extraction

### Phytochemical Screening of *E. japonicus* by Chromatographic Techniques

#### Preliminary Qualitative Phytochemical Screening by Libermann-burchards test

Total 1-mL of n-hexane extract, 1-mL of chloroform and 2 mL of acetic anhydrides, 2 mL of sulphuric acid were carefully mixed together, and an array of color changes indicated the presence of phytosterols (Table 2).

#### Phytochemical Screening of *E. japonicus* by GC-MS

The Ibn Al-Bitar Center of the Ministry of Industry and Minerals employed Agilent 19091S-433UI GC/MS equipment for GC-MS screening. The column HP-5ms Ultra Inert/60-325 350°C: 30 m\*250 m\*0.25 m was heated from 80 to 265°C at a rate of 10°C/min, the injection volume was 1 mL, the split ratio

was 1:10, the inlet temperature was 250°C, and the ionizing energy was 70.<sup>8</sup>

#### Qualitative and Quantitative Estimation of $\beta$ -sitosterol and Stigmasterol using HPLC Technique

Estimates of  $\beta$ -sitosterol and stigmasterol, both qualitative and quantitative, were made by comparing retention time of examined samples to authentic standards produced under identical chromatographic circumstances using SYKAMN (Germany) HPLC. Mobile phase HPLC conditions are as follows: Column: SYKAMN LC: C18 -ODS (25cm x 4.6 mm, 5 m particle size), Isocratic: acetonitrile: DW: acetic acid (60: 25: 5), n-hexane is an example of a sample. Standard:  $\beta$ -sitosterol and stigmasterol were measured at four different concentrations (10, 20, 30, and 40 g/mL), respectively. 1-mL per minute flow rate 100 mL injection volume, 1-mg/mL injection concentration UV Detector with a wavelength of 210 nm.<sup>9</sup>

#### Isolation and Purification of Proposed $\beta$ -sitosterol and Stigmasterol by Preparative HPLC

Rather than analysis, PHPLC is used to purify adequate quantities of a chemical for subsequent usage. It was utilized to isolate  $\beta$ -sitosterol and stigmasterol from an n-hexane extract of Iraqi *E. japonicus* L aerial portion (Germany) FOXY R1 fraction collector with S 5200 auto sampler, C18-ODS (25 cm\* 4.6 mm) column, and acetonitrile using model SYKAMN: Mobile phase: acetic acid (60: 25: 5). The isocratic elution was carried out for 10 minutes at a flow rate of 3 mL/min, using a 200 L injection volume and a UV detector set to 210 nm for detection of the isolated substance.<sup>10</sup>

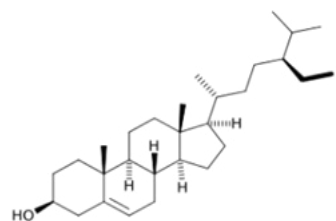


Figure 1: Beta-sitosterol structure.

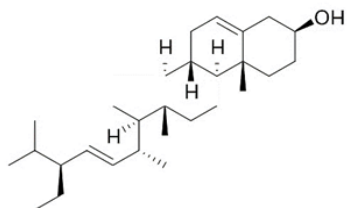


Figure 2: Stigmasterol structure.

Table 1: The reagents and materials utilized in this investigation, as well as their suppliers are listed.

Reagents and materials	Supplier
Acetic acid glacial (99.8–100%)	Analytical Rasayan/Mumbai
Acetic anhydride	BDH, Ltd. Poole/England
Acetonitrile HPLC 99.3%	Sigma-Aldrich/Germany
Analytical thin-layer chromatography (TLC) plate 0.25 mm.	MERK (Germany).
Beta sitosterol standard 90%	Chengdu Biopurify/China
Chloroform 99–99.4%	Scharlab S.L./Spain
Ethyl Acetate 99.5%	Scharlab S.L./Spain
Hexane 99%	BDH limited/England
Methanol (HPLC grade)	Sigma-Aldrich/Germany
Methanol 99.8%	Scharlab S.L./Spain
Sulfuric acid 98%	BDH limited/England
Toluene 97%	GCC/UK
Water HPLC grade $\geq 99.9$	Sigma-Aldrich/Germany

Table 2: Instruments used in the study

Instruments	Manufacturer
Chiller: Ultratemp 2000, Julabbo F30	Buchi/Germany
Electrical sensitive Balance	ADAM AFP- 360L
FTIR	IRAffinity-1-SHIMADZU- 1900 with ATR technique in BPC analysis center.
GC-MS	Agilent Technologies 7820A Ministry of Industry and Minerals, Ibn Al-Bitar Center
HPLC	SYKAMN (Germany)-column C18 in Ministry of Science and Technology, Department of Environment and Water.
Oven	MEMMERT (Germany)
Rotary evaporator	Evaporation of solvent was carried out under reduced pressure utilizing IKARV 10 D S99 R
Ultraviolet (UV-1900) spectra were recorded in methanol using a computerized spectrophotometer	SHIMADZU (Japan) in BPC analysis center
UV light–detector of 254 nm and 366 nm wavelengths.	Ultraviolet light(Desaga/Germany)
Water bath	MEMMERT (Germany)

**Table 3:** List of compounds isolated and identified through GC-MS analysis of the n-hexane extract of leaves (aerial parts) of *E. japonicus* L

No	Retention time	Iupac name	Compound	Class	Chemical formula	Pharmacological activity
1	24.502	dibutyl benzene-1,2-dicarboxylate	Dibutyl phthalate	Phthalate Ester	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	Antiandrogen (Mylchreest <i>et al.</i> , 2002), antibacterial activity <sup>12</sup>
2	30.799	bis(2-ethylhexyl) benzene-1,2-dicarboxylate	Bis(2-ethylhexyl) phthalate	Phthalate ester	C <sub>24</sub> H <sub>38</sub> O <sub>4</sub>	Behavioral Effects, Effect on Reticuloendothelial Function, <sup>13</sup> Antimicrobial <sup>9,14</sup>
3	30.799	bis(2-propylpentyl) benzene-1,2-dicarboxylate	Phthalic acid, di(2-propylpentyl)ester	Phthalate Ester	C <sub>24</sub> H <sub>38</sub> O <sub>4</sub>	antibacterial, anti-inflammatory, antioxidant, and anticancer activity <sup>15</sup>
4	30.305	(6E,10E,14E,18E)-2,6,10,15,19,23-hexamethyltetracosane	Squalene	Triterpene	C <sub>30</sub> H <sub>50</sub>	Antioxidant, <sup>16</sup> Antitumor activities, <sup>17</sup> Skin hydration <sup>13,18</sup>
5	33.503	dicyclohexyl benzene-1,2-dicarboxylate	Dicyclohexyl phthalate	Phthalate Ester	C <sub>20</sub> H <sub>26</sub> O <sub>4</sub>	Treatment of chronic inflammatory condition, <sup>19</sup> glucocorticoid receptor antagonist <sup>20</sup>
6	34.789	(2E,6E,10E)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraen-1-ol	Trans-geranylgeraniol	Sesquiterpene	C <sub>20</sub> H <sub>34</sub> O	Antibacterial, <sup>21</sup> anti-inflammatory, <sup>22</sup> anti-tumorigenic, <sup>23</sup> neuroprotective activities <sup>24</sup>

**Figure 3:** *E. japonicus*.

#### Identification and Characterization of the Isolated Compounds

The isolated stigmasterol and  $\beta$ -sitosterol was identified by using different Identification methods

- **Analytical TLC:** Toluene– ethyl acetate chloroform 5:1:4 is the optimum mobile phase, and detection is done by spraying with 10% H<sub>2</sub>SO<sub>4</sub> and heating at 110°C.
- **Fourier Transform Infrared Spectroscopy (FTIR):** is a method for obtaining an infrared spectrum of a solid, liquid, or gas's absorption or emission. An FTIR spectrometer obtains high-resolution spectral data over a large spectral range at the same time using attenuated total reflection (ATR) technique/model: SHIMADUZU1900/ range 550–4000 nm using a special standard for isolated  $\beta$ -sitosterol and stigmasterol. The isolated sample was analyzed first, followed by the standard, which was analyzed under the same conditions as the isolated material and served as a reference.

- **Ultraviolet Spectroscopy (UV):** refers to the spectroscopy of absorption or reflectance in the ultraviolet and adjacent visible parts of the electromagnetic spectrum. A UV computerized spectrophotometer model SHIMADUZU-1900 with a range of 200 to 1000 nm and a specified standard were used to analyze the UV spectrum. First, the isolated  $\beta$ -sitosterol and stigmasterol were examined, followed by the standard, which was examined under similar conditions and used as a reference.

#### RESULTS AND DISCUSSION

The active component of Iraqi *E. japonicus* L. plant extract (n-hexane extract) was identified and isolated by HPLC and RP-HPLC, and GC-MS determined the active component in the root and leaf of n-hexane extract.

#### Chemical Components Identified in Iraqi *E. japonicus* by GC-MS

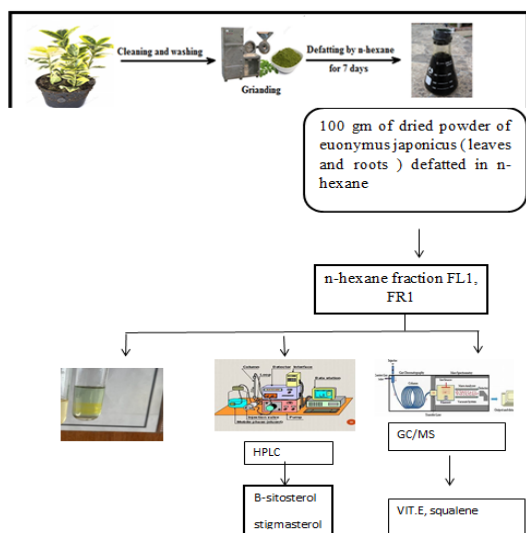
The active constituents of Iraqi *E. japonicus* L., dried aerial part and root extract, were researched in different class (sesquiterpene, triterpene, steroids). In a GC-MS analysis, 20 phytochemical compounds in the leaves part of *E. japonicus* L. and 20 phytochemical compounds in the roots were discovered. Peak area, molecular weight, and molecular formula are used to identify phytochemical substances. Some GC-MS peaks, however, remained unidentified due to a lack of legitimate samples and library data for relevant substances. Figures 4, 5, 6 and Table 3 show only six of the twenty bioactive chemicals discovered in the hexane extract of Iraqi *E. japonicus* L. leaves part. Only seven of the twenty chemicals found in the hexane extract of Iraqi *E. japonicus* L. roots are bioactive, as illustrated in Figure 7 (A, B, C) and Table 4.

**Table 4:** List of compounds isolated and identified through GCMS analysis of the n-hexane extract of *E. japonicus* L root.

No.	Retention time	Iupac name	Compound	Class	Chemical formula	Pharmacological activity
1	22.932	1,2 benzenedicarboxylic acid, bis (2-methylpropyl)ester	Diisobutyl phthalate	Phthalate ester	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	Antiandrogen (25), Estrogenic effect (26).
2	22.932	dibutyl benzene-1,2-dicarboxylate	Dibutylphthalate	Phthalate ester	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	Antiandrogen(11), antibacterial activity (12)
3	22.932	2-(heptyloxycarbonyl) benzoic acid	Monoheptyl phthalate	Phthalate ester	C <sub>15</sub> H <sub>20</sub> O <sub>4</sub>	antagonist progesterone receptor.
4	31.650	2 <i>R</i> )-2,5,7,8-tetramethyl-2-[(4 <i>R</i> ,8 <i>R</i> )-4,8,12-trimethyltridecyl]-3,4-dihydrochromen-6-yl] acetate	Alpha tocopherol acetate	Tocopherol	C <sub>31</sub> H <sub>52</sub> O <sub>3</sub>	treatment of pollen-induced allergic rhinitis(27), antioxidant activity(28)
5	31.650	(2 <i>R</i> )-2,5,7,8-tetramethyl-2-[(4 <i>R</i> ,8 <i>R</i> )-4,8,12-trimethyltridecyl]-3,4-dihydrochromen-6-ol	Vit E	Tocopherol	C <sub>29</sub> H <sub>50</sub> O <sub>2</sub>	Antioxidant(29), <u>improve insulin action</u> (30)
6	33.267	(6 <i>E</i> ,10 <i>E</i> ,14 <i>E</i> ,18 <i>E</i> )-2,6,10,15,19,23-hexamethyltetracosane	Squalene	Triterpene	C <sub>30</sub> H <sub>50</sub>	Antioxidant(16) , Antitumor activities(17) , Skin hydration (18)
7	23.679	2-(pentylloxycarbonyl) benzoic acid	Monopentyl Phthalate	Phthalate ester	C <sub>13</sub> H <sub>16</sub> O <sub>4</sub>	Antiandrogen (31)

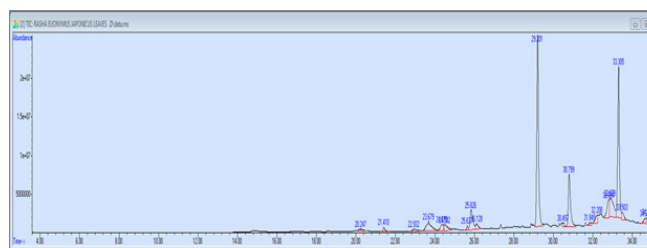
**Table 5:** Characteristic infra-red frequencies of isolated  $\beta$ -sitosterol and stigmasterol compound.

Phytosterols compound	Approximated position of characteristic bands (cm <sup>-1</sup> )
$\beta$ -Sitosterol	3421.72 (free O-H stretching band), 2943.37 and 2858.54 (aliphatic C-H stretching), 1647.21 (C=C absorption peak), 1458.18 (C-H bending/-CH <sub>2</sub> ), 1056.99 (cycloalkane), 956.11 C-H out plane bending
Stigmasterol	3425.58 (free O-H stretching band), 2935.52 and 2862.36 (aliphatic C-H stretching), 1558.48 (C=C stretching), 1373.3 (CH <sub>2</sub> -CH <sub>3</sub> stretching) 1022.72 (C-H bending)

**Figure 4:** Step of extraction of plant *E. japonicus*.

### Identification and Quantification of $\beta$ -sitosterol and Stigmasterol by HPLC

By comparing the retention reference standards under identical chromatographic conditions, the HPLC method was employed to detect various compounds. HPLC is a fast, easy, and reliable method for detecting compounds. HPLC was an effective



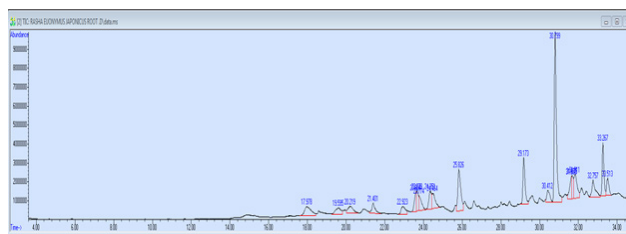


Figure 6: GC/MS for the n-hexane fraction of *E. japonicus* L dried root part.

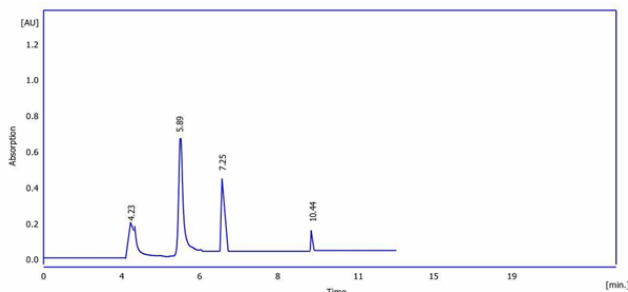


Figure 7 (A): HPLC analysis for n-hexane fraction of Iraqi *E. japonicus* L. dried leaves part.

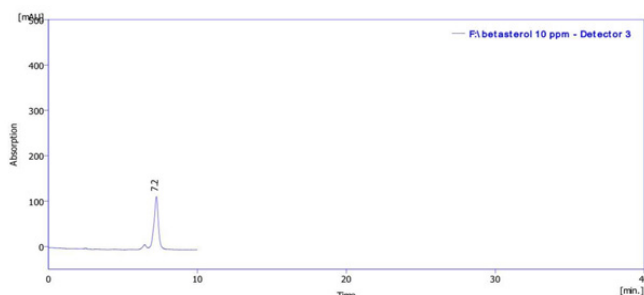


Figure 7 (B): HPLC for beta-sitosterol standard.

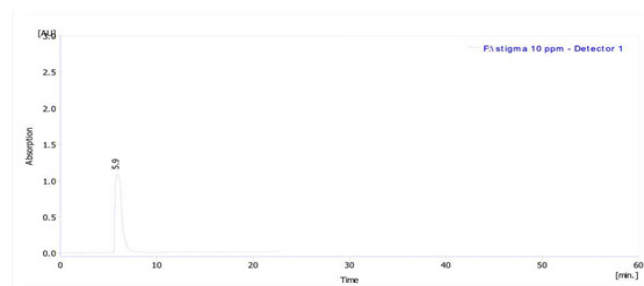


Figure 7 (C): HPLC for stigmasterol.

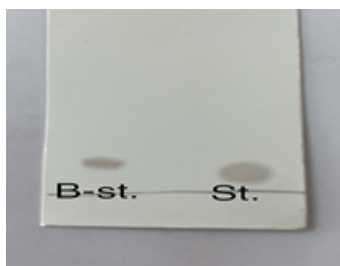


Figure 8: Thin layer chromatogram of separated  $\beta$ -sitosterol from n-hexane fraction with  $\beta$ -sitosterol standard, produced in toluene-ethyl acetate-chloroform 5:1:4 as a mobile phase and detection by spraying with 10%  $H_2SO_4$  + heating at 110°C

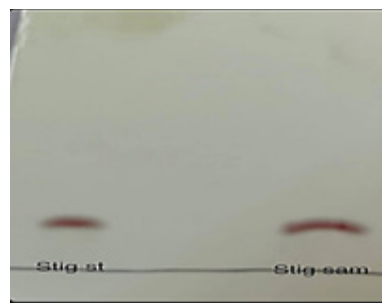


Figure 9: Thin layer chromatogram of the isolated stigmasterol from n-hexane fraction with stigmasterol std. using silica gel GF254nm as a stationary phase developed in toluene-ethyl acetate-chloroform 5:1:4 as a mobile phase and detection by spraying with 5%  $H_2SO_4$  + heating at 110°C.

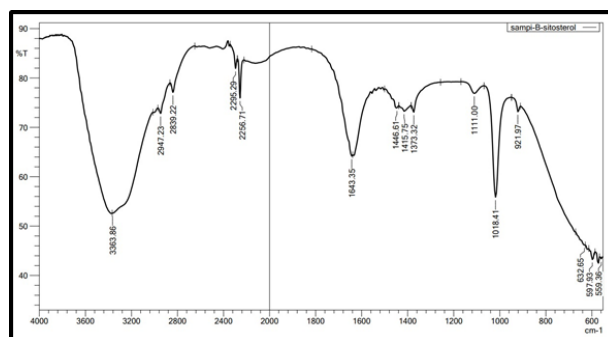


Figure 10 (A): FTIR spectra isolated  $\beta$ -sitosterol.

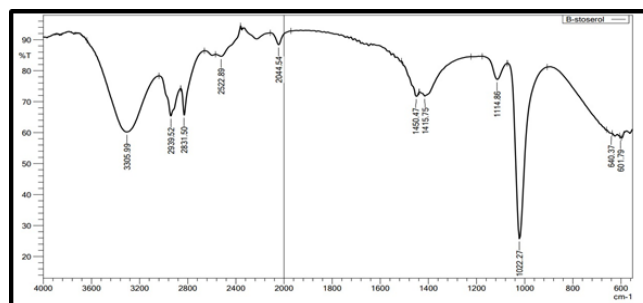


Figure 10 (B): FTIR spectra  $\beta$ -sitosterol standard.

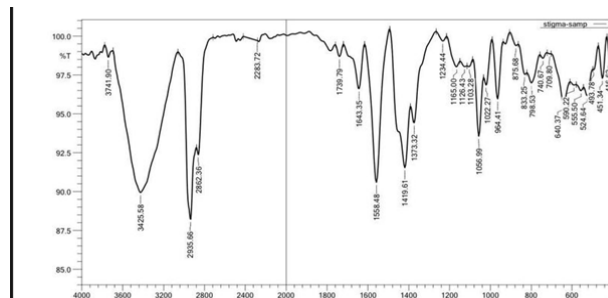


Figure 11 (A): FTIR spectra of stigmasterol sample.

( $\beta$  -sitosterol) at retention time is a prominent peak that was identified by comparison with standard  $\beta$ -sitosterol and stigmasterol at retention time. After being monitored according to the time (time from peak appearance to peak disappearance) in Figure 7 (A-C), the major peak was collected by the fractions

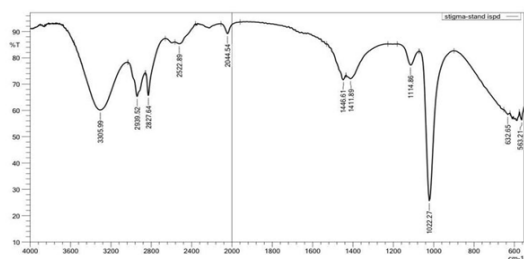


Figure 11 (B): FTIR spectra of stigmasterol standard.

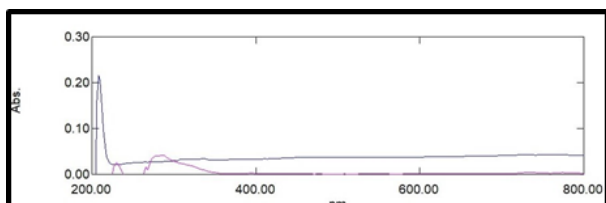


Figure 12: UV spectrum of the  $\beta$ -sitosterol std. and the isolated  $\beta$ -sitosterol from the n-hexane fraction.

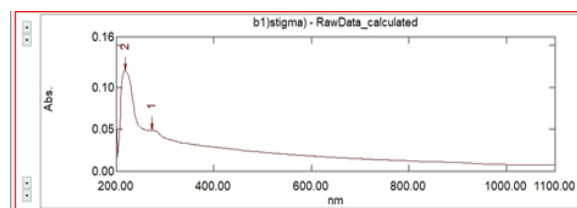


Figure 13 (A): UV spectrum of stigmasterol sample.

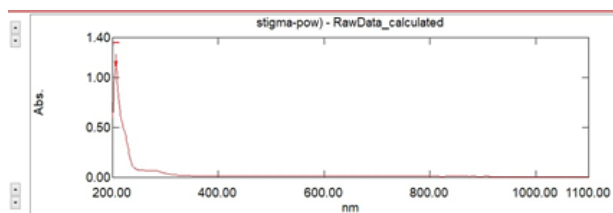


Figure 13 (B): UV spectrum of stigmasterol standard.

collector, and the sample obtained from P-HPLC was dried over anhydrous sodium sulfate, weighted, and subjected to various identification methods.

### Characterization of the Isolated $\beta$ -sitosterol

#### TLC

As shown in Figure 9, the isolated compound appeared as a single spot with the same color and  $R_f$  value as standard  $\beta$ -sitosterol and stigmasterol.<sup>10</sup>

#### Fourier Transforms Infrared (FTIR) Spectra

Because FTIR spectroscopy is most commonly used in phytochemical studies as a finger printing device for comparing a natural with a synthetic reference standard, the IR spectra of the isolated compound gave identical results when compared to standard  $\beta$ -sitosterol, as shown in Figure 10 (A and B), and 11 (A and B).

#### Ultraviolet Spectroscopy (UV)

The UV spectrum was recorded between 200 and 600 nm, and the results revealed that the maximum absorbance spectrum

of the  $\beta$ -sitosterol and stigmasterol standard and the isolated product were the same at the same wavelength, as illustrated in Figures 12, and 13 (A, B).

### CONCLUSION

The existence of a substantial set of natural therapeutic compounds belonging to several chemical classes was discovered by GC-MS phytochemical study of Iraqi *E. japonicus* L. (leaves and root). The quantity and quality of these natural medicinal ingredients vary from the leaves to the root. HPLC was utilized to isolate and purify  $\beta$ -sitosterol and stigmasterol from the n-hexane fraction of a leaves part, followed by a simple and repeatable TLC and HPLC approach, and finally, FTIR and UV.

### ACKNOWLEDGMENT

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